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TITLE: Characterization of the Hematopoietic Stem Cell in the Peripheral Blood of Patients with Idiopathic Myelofirosis

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14. ABSTRACT
The clinical course of patients with Philadelphia chromosome negative myeloproliferative disorder (MPD) is frequently complicated by thrombotic events. Post-natal vasculogenesis has been proposed to play a critical role in angiogenesis by acting through a hierarchy of endothelial progenitor cells (EPC). Some EPC have been shown to share a number of features associated with monocytes while other more primitive progenitor cells produce EC in vitro exclusively. The cells which share features of monocytes and endothelial cells have been termed angiogenic monocytes (AM). Reduced levels of AM progenitor cells have been reported to be predictive of atherosclerotic disease progression. AM progenitor cells were assayed in vitro from the peripheral blood mononuclear cells (MNC) of MPD patients. AM colonies were plucked and analyzed for EC and hematopoietic cell markers, JAK2V617F and their ability to incorporate into vascular endothelium following their transplantation into immunodeficient mice (NODISCID). MPD AM colonies that were detected were uniformly JAK2V617F positive and produced cells that expressed phenotypic markers characteristic of both monocytes and EC. Reduced numbers of AM colonies were present in the blood of MPD patients with a high JAK2V617F burden (>50%), (p<O.Ol). Transplanted AM were able to contribute to the vascular endothelium of NODISCID mice. These studies suggest that reduced numbers of circulating AM progenitors contribute to the propensity to develop thrombotic complications in MPD patients.

15. SUBJECT TERMS
endothelial cells, angiogenic monocytes, Myeloproliferative disorders, Thrombosis

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Table of Contents

Introduction:

Vascular endothelium regulates a host of processes which provides a non-adhesive surface to circulating neutrophils and platelets while helping to prevent the blood clotting. A number of groups have hypothesized that endothelial cell (EC) dysfunction might contribute to the hypercoagulable state associated with Ph^- MPD by orchestrating the recruitment of blood elements to sites of injury or regulating vascular tone by impairing the release of nitric oxide. During early mammalian development a common cell of origin for hematopoietic and endothelial cell elements has been identified. This so call hemangioblast has been characterized in adults but its role in normal homeostasis or disease biogenesis remains poorly defined. Since 1997, post-natal vasculogenesis has been proposed to play a critical role in angiogenesis by acting through a hierarchy of endothelial progenitor cells (EPC). Some of these so called EPC have been shown to share a number of features associated with monocytes while other more primitive progenitor cells produce EC in vitro exclusively. The cell which shares features of monocytes and endothelial cells have been termed angiogenic monocytes (AM). AM likely do not give rise to new vessels but are thought to recruit or facilitate EC required for vessel repair and maintenance of the integrity of the endothelium. Reduced levels of AM progenitor cells have been reported to be predictive of atherosclerotic disease progression.

Body:

Colonies of AM generated *in vitro* from MPD MNC:

AM colonies were generated by culturing mononuclear cells (MNC) isolated from the PB of normal controls (NC; n=7) and patients with MPD (total n=41; PV, n=18; primary myelofibrosis, PMF; n=14)

AM containing colonies were identified by their morphological appearance; a central cluster of round cells and radiating elongated spindle-like cells at the periphery which after reaching confluence form areas which resembled cobblestones. The period of incubation required for the AM to reach confluency was greater for MPD MNC as compared to NC (21±2 vs. 12±2 days). HUVEC expressed exclusively EC markers: $CD31^+$, $CD144^+$, $VEGFR2^+$, $ULEX\text{-}1^+$, vWF^+, $CD105^+$, $CD45^-$ and $CD14^-$ while cells within AM colonies assayed from normal and MPD MNC co-expressed EC and myeloid markers including $CD31^+$, $CD144^+$, $VEGFR2^+$, $ULEX\text{-}1^+$, vWF^+, $CD105^+$, $CD45^+$ and $CD14^+$. Both NC and MPD AM irrespective of JAK2V617F status were also able to take up acetylated low density lipoprotein (Ac-LDL).

The number of AM containing colonies formed by NC, PV and PMF MNC ($1x10^6$) was determined. PMF MNC formed greater numbers of AM containing colonies than NC or PV MNC (169.5± 80 vs. 83±20 or 63.1±64.5 *p<0.01*),(Figure 1).

MNC from JAK2V617F negative PMF patients formed greater numbers of AM containing colonies than NC MNC (216.8±42.8 vs. 83±20.6; *p<0.01)* By contrast, MNC from PMF patients who were JAK2V617F positive (high burden (>50%) and low burden(<50%) allele formed fewer AM colonies than the JAK2V617F negative PMF (45.25±25.2 *p<0.01* and 143.08±25.3 vs. 169.5± 80 *p<0.05*) In addition, MNC from PV patients with a high burden of the JAK2V617F allele formed fewer AM containing colonies than MNC from NC (29.2±42.8 vs. 83±20.6; *p<0.01*) or PV patients with a low burden of JAK2V617F (105.4±63.9; *p<0.01*).The numbers of AM colonies were similarly reduced when MNC from patients with either high allele burden PV or PMF were assayed.

A.

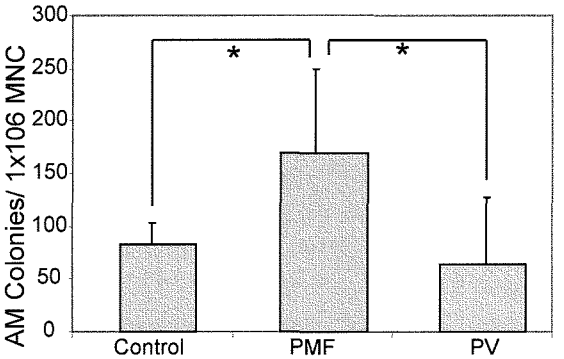

Figure 1: Number of AM containing colonies assayed from MNC of normal controls (n= 7), PMF (n=14), PV (n=18) * = p<0.01.

Analysis of JAK2V617F in PV AM containing colonies: (Figure 2):

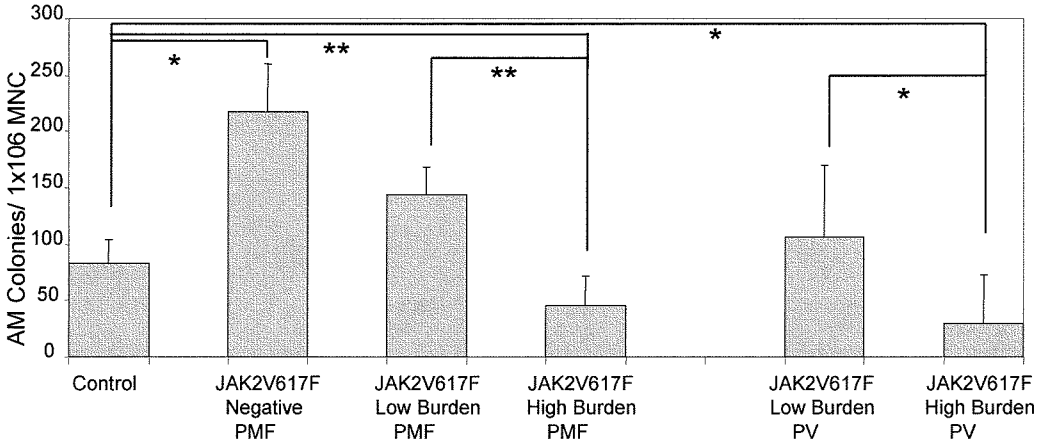

Figure 2: Number of AM containing colonies assayed from MNC of normal controls (n= 7), JAK2V617F negative PMF (n=9), low JAK2V617F burden PMF (n=2) and high JAK2V617F burden PMF (n=3) and low JAK2V617F burden PV (n=8) and high JAK2V617F burden PV (n=10). * = $p<0.01$; **= $p<0.05$

The AM containing colonies assayed from 5 patients with JAK2V617F positive PV were plucked and analyzed for JAK2V617F by nested-PCR. Among the 67 AM containing colonies analyzed, 72.2% of the colonies were homozygous for JAK2V617F 22.2% were heterozygous for JAK2V617F and 5.5% contained exclusively wild type (wt) JAK2. JAK2V617F homozygous AM containing colonies were assayed from PV MNC of each of the 4 PV patients studied while 2 of the 4 patients also had a significant number of AM containing colonies with the WT JAK2. In each of the 4 patients studied the number of AM containing colonies homozygous for JAK2V617F exceeded the number either of heterozygous JAK2V617F or WT JAK2 AM containing colonies.

We compared the percentage of homozygous, heterozygous JAK2V617F and WT JAK2 AM containing colonies and the percentage of hematopoietic cell colonies with the corresponding JAK2 status. MNC were assayed from two patients with low burden JAK2V617F PV and two patients with high burden JAK2V617F PV. In the patients with a high burden of JAK2V617F both the hematopoietic and AM containing colonies were predominantly homozygous for JAK2V617F while in the low burden patients a greater proportion of the AM containing colonies than HC colonies were homozygous for the mutation.

MPD AM engraft NOD/SCID mice:

We attempted to assess if AM generated in vitro from MPD MNCs contribute to vascular endothelium by transplanting AM cells into NOD/SCID mice. Cord Blood (n=6), NC (n=3), PV (n=10) or PMF (n=3) AM generated in vitro from MNC were harvested after 14 days of culture and labeled with CFSE. Cells were then transplanted into sublethally irradiated NOD/SCID mice. After 7 days, nonhematopoietic organs (heart, lung, liver, spleen) were harvested, sectioned and analyzed using confocal and fluorescent microscopy to detect the CFSE positive cells within vessel walls. A total of 5 sections were examined from each nonhematopoietic organs harvested from the transplanted mice. In 50% of the animals transplanted with CB AM, 100% of animals transplanted with NC AM, 60% of the animals transplanted with PV AM and 100% of the animals transplanted with PMF AM CFSE positive cells were observed in multiple sections of liver, lung, heart and spleen. Cells were seen within the endothelium and subendothelial space of vessels appearing as cells with relatively small round nuclei demonstrating green birefringence of the cytoplasm (Figure 3).

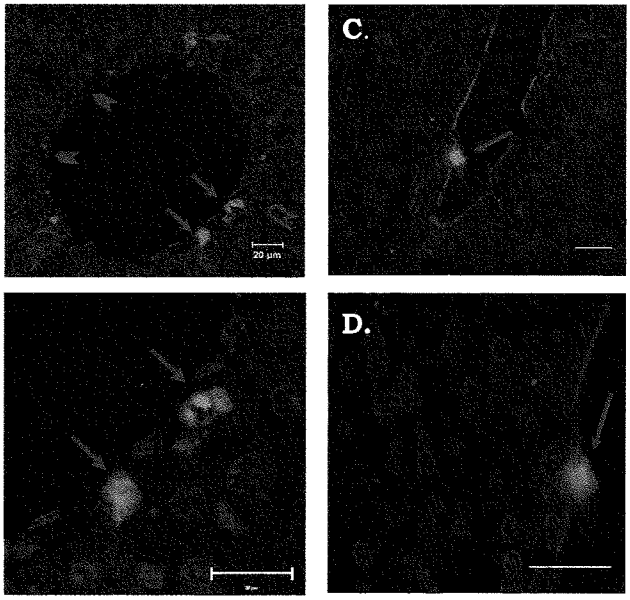

Figure 3: MPD AM containing colonies at the end of 14 days culture were harvested and labeled with CFSE. Labeled cells then transplanted into sublethally irradiated NOD/SCID mice. After 7 days, EC rich organs were harvested and sections were analyzed using fluorescent or confocal microscopy to detect the engrafted MPD AM cells.

A. Centrilobular zone of one transplanted NOD/SCID mouse showing a terminal hepatic venule (THV) in the center. The cells lining the venule have spindle or oval shaped nuclei representing endothelial cells (broad arrowheads). Arrows point to two cells along the endothelial lining that are positive. A single + cell that appears to be approaching the THV is also seen along the sinusoids (open arrowhead). (DAPI stain, Original magnification x 400)

B. High power magnification of the two + cells in A shows that the staining is in the cytoplasm with a predominantly perinuclear location. (DAPI stain, Original magnification x 800)

C. A low power photomicrograph showing another THV on another NOD/SVID Mouse with a positively-stained cell along the endothelial lining. (DAPI stain, Original magnification x400)

D. High power magnification demonstrates cytoplasmic reactivity of an endothelial cell (arrow). The positive fluorescence in the cytoplasm partially covers the nucleus of the cell which now appears pale blue and smudged. (DAPI stain, Original magnification x 800)

Similar cells were seen in the sinusoids approaching a terminal hepatic venule. These studies indicate that PV and PMF AM are capable of being integrated into EC layers or subendothelial areas of vessels within the livers, hearts, spleens and lungs of NOD/SCID mice.

Key Research Accomplishments:

- Reduced numbers of AM progenitor cells in patients with high burden JAK2V617F positive MPD likely contributes to the propensity of MPD patients to develop thrombotic complications by limiting their capacity to repair injured endothelium.
- The number of assayable circulating AM might serve as a biomarker for thrombotic risk in patients with MPD.
- MPD AM home to injured endothelium.

Reportable Outcomes:

Selcuk Sozer, Xiaoli Wang, Wei Zhang, Maria Isabel Fiel, Takefumi Ishii, Jiapeng Wang, Nathaniel Wisch, Mingjiang Xu and Ronald Hoffman; "Circulating Angiogenic Monocyte Progenitor Cells Are Reduced in JAK2V617F High Allele Burden Myeloproliferative Disorders"; Blood Cells, Molecules and Disease. In Press 2008

Special Note:
The pursuit of the research plan was delayed due to PI relocated from University Illinois Chicago to Mount Sinai School of Medicine. The research resumed as of December 2007.